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Novel Epidermal Growth Factor Receptor Drug Platforms

Aneliya Zdravkova Rankova
Worcester Polytechnic Institute

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Novel Epidermal Growth Factor Receptor Drug Platforms

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Aneliya Rankova

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APPROVED:

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Joseph B. Duffy, Ph.D.
Biology and Biotechnology
WPI Project Advisor

Abstract

Aberrant signaling by the epidermal growth factor receptor (ErbB) family plays a central role in tumor development and is a target for pharmacological intervention. A major challenge in drug discovery is the evaluation of new compounds at the organismal level. Due to conservation of the ErbB network between *Drosophila* and humans, flies offer an attractive system for this. I have generated transgenic *Drosophila* with human ErbB receptors and used them to develop an *in vivo* assay to screen for therapeutics.

Acknowledgements

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Introduction

Cancer is currently the second leading cause of death in the western world and its global health burden continues to increase at an alarming pace. One in every eight deaths worldwide is caused by cancer – more than AIDS, malaria and tuberculosis combined. It is estimated that in 2008 alone, there were 7.4 million deaths from cancer and 12.4 million new diagnosed cases around the world (American Cancer Society, 2010). Advances in diagnosis and treatment have increased patient survival in several types of solid tumors. However, drug resistance and treatment-related toxicity still remain a major cause for the overall poor patient outcome associated with cancer. Consequently, there is an increasing need to develop more effective treatments to improve patient survival (Madhusudan & Ganesan, 2004).

Advances in our understanding of the biology of cancer have led to the identification of receptor tyrosine kinases (RTK), a class of transmembrane receptors, coupled to complex signal transduction networks that regulate critical cellular processes such as survival, proliferation, differentiation and migration. Aberrant signaling by RTKs is the hallmark of human oncogenesis and has emerged as a major mechanism for tumor cell proliferation and survival. Consequently, RTKs have provided a new arena for oncology drug discovery, where a novel class of therapeutic compounds is developed to specifically block or attenuate RTK activity (Lemmon & Schlessinger, 2010).

The Epidermal Growth Factor Receptor Family of RTKs

The epidermal growth factor receptor family is a subclass of RTKs and comprises four members: ErbB1/EGFR, ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. ErbB1 was the first RTK to be discovered and subsequently provided the first relationship between an oncogene and cancer. Upon ligand binding, the receptors form homo and heterodimeric complexes to initiate intricate signal transduction cascades that relay information to the nucleus and control a diverse array of cellular processes including differentiation, proliferation, apoptosis and migration(Bazley & Gullick, 2005).

The ErbB receptors and their pivotal role in cell fate determination appear to be evolutionary conserved. *Drosophila melanogaster* has an ErbB homolog named DER, which shares many structural and functional similarities with the human receptors. The overall amino acid identity between ErbB1 and DER is 38% (Bogdan & Klambt, 2001). EGFR signaling in vertebrates is essential for normal development as demonstrated by studies in genetically modified mice. Loss of EGFR signaling causes embryonic or perinatal lethality in mice and abnormalities in multiple organs including brain, skin, lung heart and gastrointestinal tract. In addition, the EGFR receptors promote mammary gland development in the adult (Olayioye, et al., 2000).

Domain Architecture of the ErbB Family

The four members of the EGFR family are closely related transmembrane glycoproteins that contain three functional regions: an extracellular region for ligand binding and receptor dimerization, a single transmembrane domain and a cytoplasmic region (Fig. 1) (Bazley & Gullick, 2005).

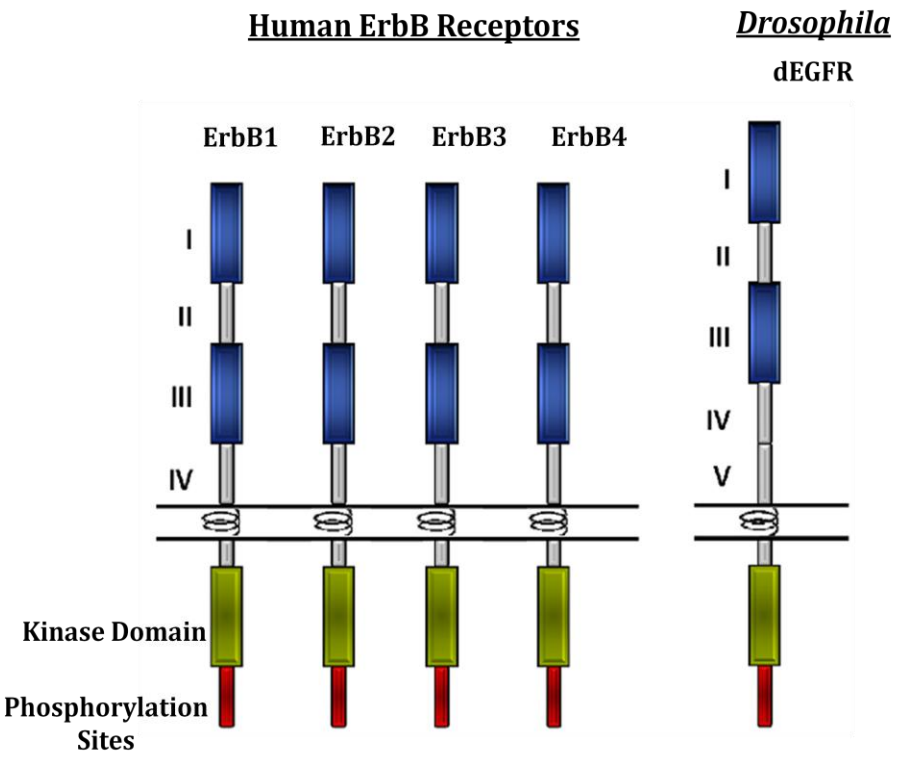


Figure 1: Structure of the *Drosophila* and Human Epidermal Growth Factor Receptors

The extracellular region of the four ErbB receptors consist of two homologous large domains (I and III) and two cysteine-rich domains (II and IV). Domains I and III are important for ligand binding, while receptor dimerization is mediated principally by domain II. In addition, structural studies have identified a small region within domain IV that is also involved in physical receptor-receptor interactions (Fig. 1) (Burgess et al., 2003).

The cytoplasmic region of the EGFR receptors contains three distinct domains: the juxtamembrane domain that interacts with protein kinase C (PKC), the non-catalytic carboxy-terminal tail housing the tyrosine transphosphorylation sites and the tyrosine kinase domain, which transphosphorylates carboxy-terminal tyrosine residues (Blazley et al).

Ligand Binding and Receptor Activation

The ErbB receptors are activated by members of the EGF family of peptide growth factors which include EGF, transforming growth factor-alpha (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), epigen (EPG), and the neuroglins (Fig. 2) (Wilson et al., 2009).

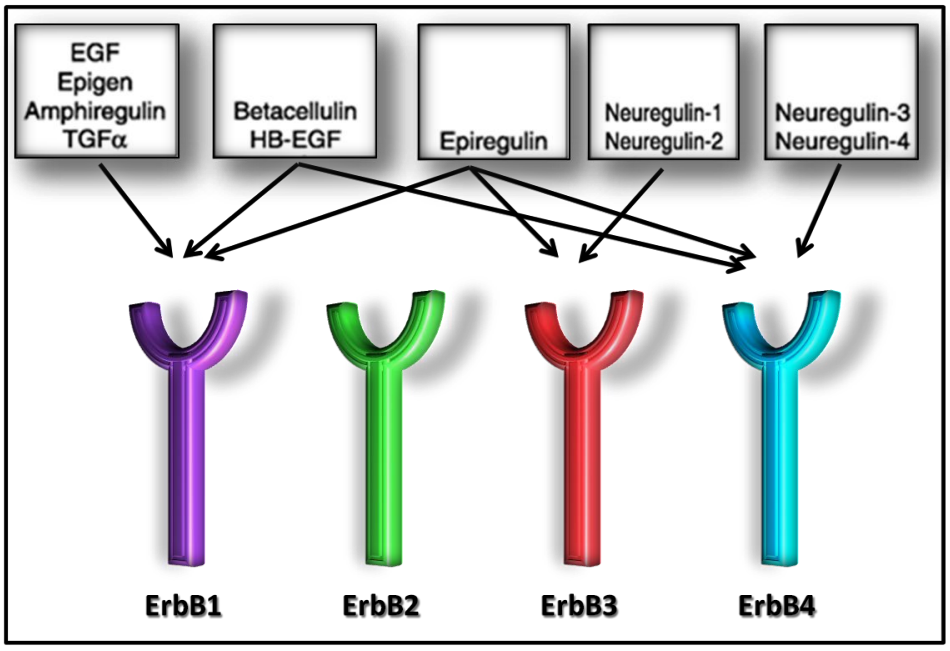


Figure 2: EGF ligand family bind and activate multiple EGFR receptors.

In the absence of ligands the ErbB receptors exist as inactive tethered monomers due to direct intramolecular interactions between domains II and IV. By binding simultaneously to both domains I and III, the ligand induces substantial conformational rearrangements of the extracellular region, exposing a dimerization arm in domain II (Fig. 3). These conformational changes promote receptor homo- and heterodimerization and subsequent activation of the intracellular kinase domain. Each receptor in the dimer transphosphorylates specific tyrosine residues on the C-terminal domain of its partner. The resulting phosphotyrosines then become docking sites for various signaling molecules initiating a diverse array of downstream signaling pathways (Burgess et al., 2003).

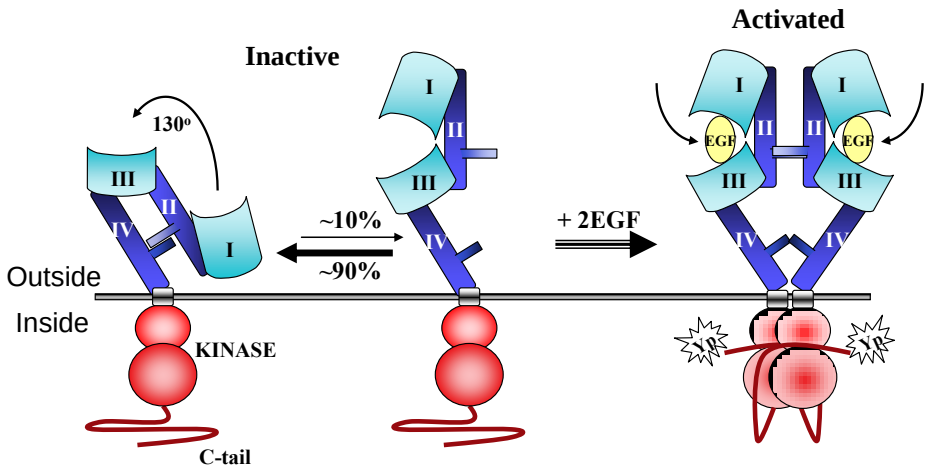


Fig. 3: Ligand-Induced Receptor Dimerization

Two of the ErbB receptors possess unique characteristics that distinguish them from the rest of the ErbB family members. ErbB3 has a defective intracellular kinase domain and is incapable of phosphorylating the C-terminal tyrosine residues of its dimerization partners. The second member of the family, ErbB2, has no known ligand that interacts with its extracellular domains. Nevertheless, ErbB2 is capable of initiating potent signaling cascades upon dimerization and is the only receptor in the family that can transform cells when simply overexpressed (Burgess et al., 2003). Structural studies revealed that unliganded ErbB2 lacks the autoinhibitory domain II/IV tether interactions and adopts a constitutively extended extracellular configuration with its dimerization arm exposed. This

extracellular domain arrangement resembles the active conformation of the rest of the ErbB family members and, strikingly, the unliganded conformation of the *Drosophila* ErbB homolog, DER (Alvarado et al., 2009).

Unlike the orphan ErbB2, four ligands (Spitz, Gurken, Keren and Vein) tightly regulate DER and are required for receptor activation and dimerization. This suggests that autoinhibitory interactions, different from those present in ErbB1, ErbB3 and ErbB4, keep unliganded DER in an inactive conformation. Although the dimerization arm of DER is exposed in the absence of a ligand, direct interactions between domains I and III keep the dimerization arm in an orientation that inhibits receptor dimerization. Ligand binding pushes domains I and III apart from each other causing domain II to bend and reorient its dimerization arm. The resulting rearrangements are necessary for receptor-receptor interactions. The structural similarities between the inactive DER and ErbB2 together with fact that ErbB2 has more propensity to form heterodimers than homodimers, suggest that ErbB2 possibly utilizes a similar autoinhibitory mechanism (Alvarado et al., 2009).

Downstream Signaling Pathways

Activated receptor dimers often utilize the mitogen activated protein kinase (MAPK) pathways to relay information to the nucleus. The MAPK pathway is a highly conserved signaling module that regulates fundamental cellular processes such as proliferation, differentiation and survival. Mammalian cells utilize four MAPK pathways that consist of three enzymes: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK. Among the four MAPK pathways, the RAF-MEK-ERK signaling pathway is the most extensively studied, reflecting its established role in tumor development (Fig. 4). Aberrant activation of the cascade can occur through either a ligand-induced signaling by receptor tyrosine kinases or mutations and overexpression of the proteins that constitute the pathway (Montagut & Settleman, 2009).

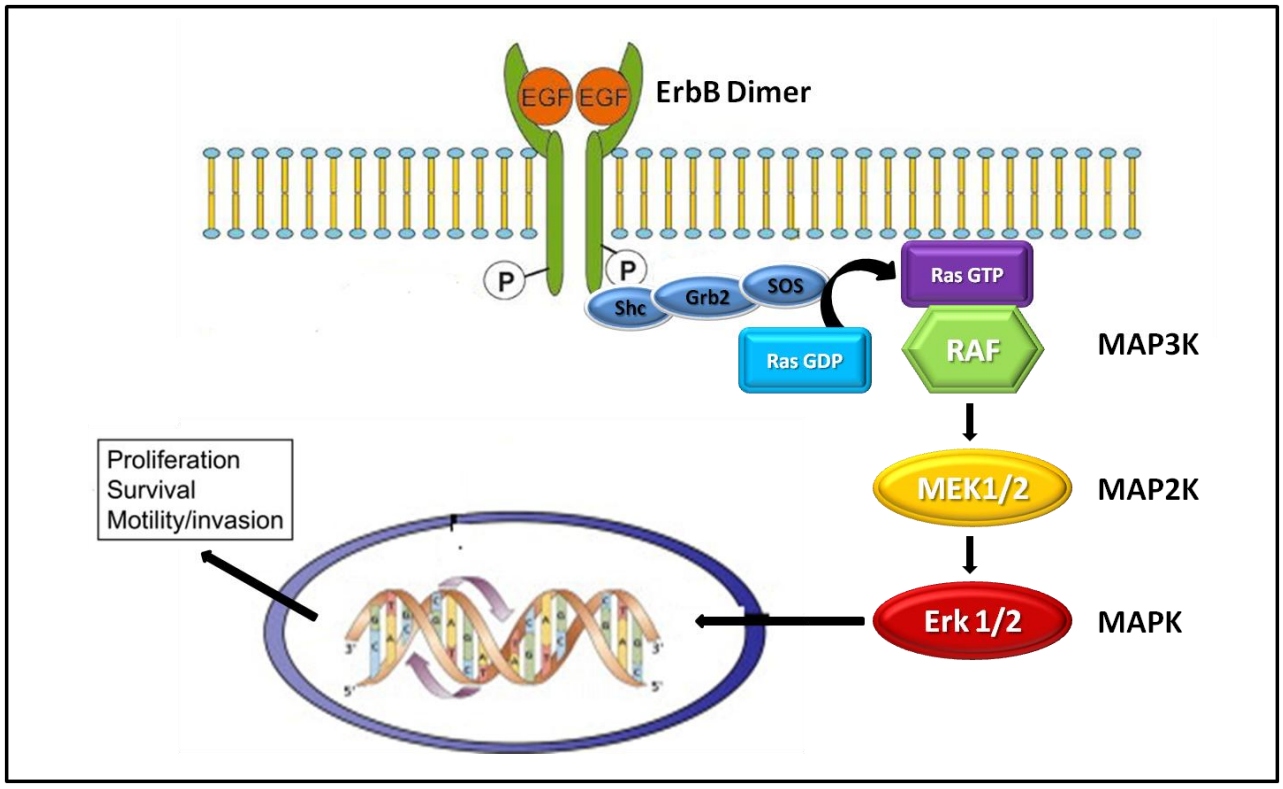


Fig. 4: ErbB Mediated Activation of the MAPK Pathway.

Receptor dimerization and subsequent phosphorylation of specific tyrosine residues at the C-terminal tail create docking sites for various second messenger molecules that link activated receptors to distinct downstream signaling cascades. These second messenger molecules can be either adaptor proteins or enzymes that bind to phosphotyrosine residues via SH2 (src homology) or PTB (phosphotyrosine binding) domains (Prenze et al., 2001). Two adaptor proteins, Shc (src-homology 2 containing) and Grb2 (growth factor receptor bound-2) provide a link between the active ErbB1 receptor and the MAPK signaling pathway (Fig. 4). Shc binds directly to phosphotyrosine residues of the active receptor and recruits Grb2 which in turn associates with the guanine nucleotide exchange factor SOS (son-of-sevenless). SOS activates the small G-protein Ras by exchanging GDP for GTP. Ras then undergoes a conformational change that allows it to interact with the serine/threonine kinase Raf. This results in Raf translocation to the cell membrane where its activation takes place. Raf then activates the tyrosine and serine/threonine dual specificity kinase MEK by phosphorylating S residues in its catalytic domain. MEK in turn

phosphorylates and activates the serine/threonine kinases ERK1/2 (extracellular-signal-activated-kinases, also known as MAPK) which can translocate to the nucleus and directly activate transcriptional factors including Ets-1, c-Jun and c-Myc (McCubrey et al., 2006).

Regulation of Receptor Signaling

Following ligand binding and subsequent receptor activation, the ErbB dimers are targeted for endocytosis in clathrin-coated membrane invaginations, which pinch off to form vesicles that deliver the receptors to endosomes. Internalized receptors are either targeted for lysosomal degradation or recycled back to the plasma membrane. This mechanism regulates the mitogenic potency of the ErbB receptors through modulating signal strength and duration early in the signaling pathway (Sorkin & Goh, 2009).

Translocation of active receptor dimers to endosomes is followed by recruitment of the E3 ubiquitin ligase Cbl, which associates with specific phosphotyrosines at the C-terminal tail of the receptors. The mammalian family of Cbl proteins comprises three members, c-Cbl, Cbl-b and Cbl-3, which directly bind specific ErbB phosphotyrosines via their tyrosine kinase binding (TKB) domains. In addition, the Cbl proteins contain a RING Finger domain that recruits E2 ubiquitin conjugating enzymes. Thus, Cbl targets internalized receptors for lysosomal degradation by promoting receptor ubiqiutination (Sorkin & Goh, 2009).

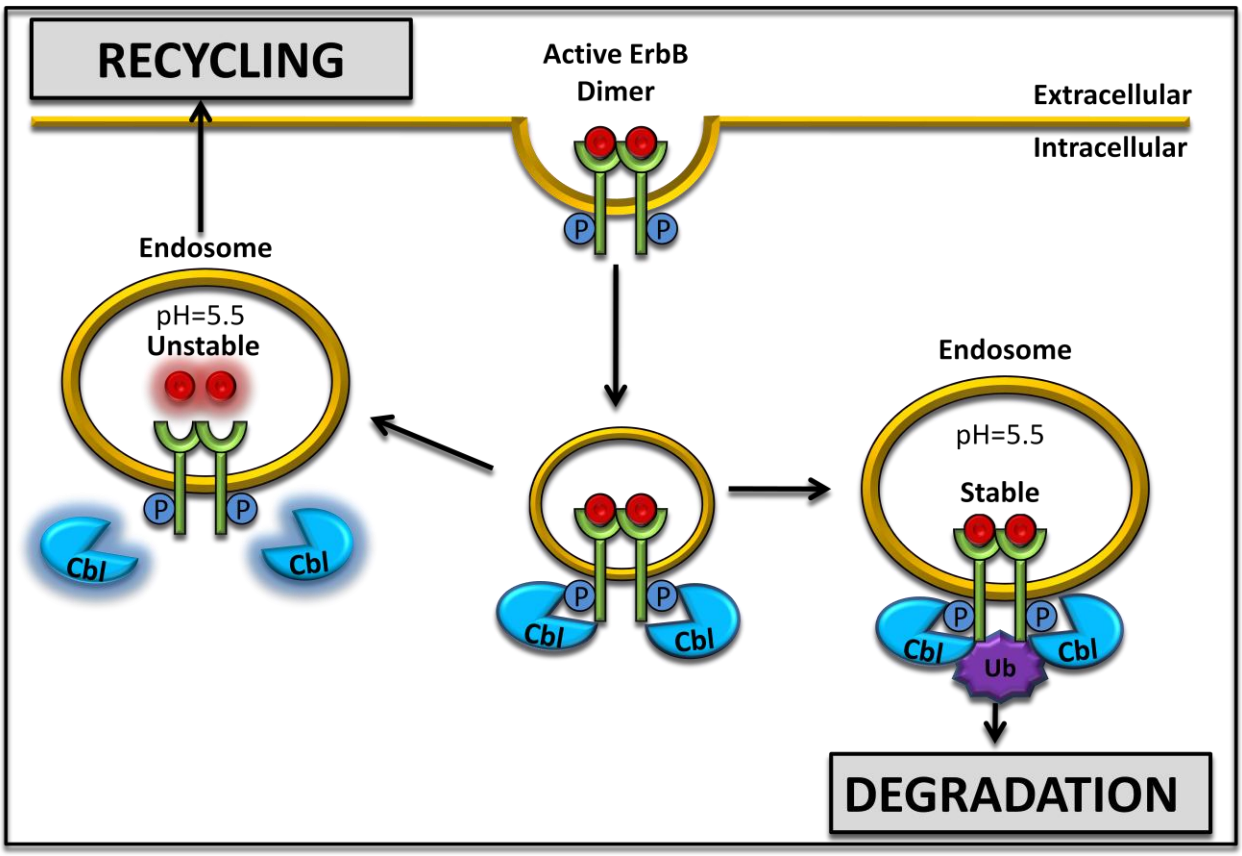


Figure 5: Ligand-induced receptor endocytosis.

Another determinant of receptor fate following endocytosis is the stability of the activated receptor-ligand complex. In the slightly acidic endosomal environment receptor homodimers remain stably bound to their ligands, which promotes receptor ubiquitination by Cbl and subsequent lysosomal degradation. By contrast, the ErbB heterodimers are less stable in the acidic endosomal environment causing the ligand and Cbl to dissociate. As a result, the receptors are recycled back to the cell membrane (Fig. 5). This differential endocytinc routing of receptor dimers partially accounts for the enhanced mitogenic potency of heterodimers (Lenferink et al., 1998).

Epidermal Growth Factor Receptors in Cancer

In normal cells, the potent mitogenic signals generated by the ErbB receptors are tightly regulated through mechanisms such as receptor endocytosis to ensure proper interpretation of the extracellular signals. On the other hand, dysregulation of the ErbB signaling pathways contributes to uncontrolled cell proliferation and has been implicated

in the development and progression of multiple types of cancer including breast, lung, brain and head and neck cancer (Table 1). Hyperactivation of the ErbB network in cancer is usually the result of the following three mechanisms: overexpression of receptors as a result of gene amplification or polysomy, overproduction of receptor ligands, or somatic mutations that lead to the production of receptors that are constitutively active even in the absence of a ligand (Yarden & Sliwkowski, 2001).

Table 1: ErbBs expression in cancer (Yarden & Sliwkowski, 2001)

Receptor	Type of Dysregulation	Type of Cancer
ErbB1	Overexpression	Head and neck, breast, bladder, prostate, kidney, lung, glioma
	Mutation	Breast, glioma, lung, ovary
ErbB2	Overexpresion	Breast, lung, pancreas, colon, oesophagus, endometrium, cervix
ErbB3	Expression	Breast, colon, gastric, prostate
	Overexpression	Oral squamous cell cancer
ErbB4	Expression	Childhood meduloblastoma

Overexpression and structural changes in the ErbB receptors are both implicated in the development of human malignancies. However, *in vitro* studies demonstrate that overproduction of normal receptors causes cell transformation only in the presence of appropriate levels of ligands. For instance, overexpression of ErbB1 in mouse NIH-3T3 cells leads to transformation of these cells only upon addition of EGF, which is necessary for receptor activation. Increased levels of ErbB1 expression occur in 40% of human gliomas and are associated with poor patient outcome. In addition, a specific type of ErbB1 mutation that results in constitutively active receptor due to deletions in the extracellular domains has been identified in ovary and breast carcinomas (Normanno et al., 2006).

Deregulated signaling by the ErbB1 receptor has also been associated with the formation and pathogenesis of non-small cell lung carcinoma (NSCLC), which is characterized by a particularly adverse clinical prognosis. NSCLC is the leading cause of cancer death

worldwide accounting for one third of all cancer deaths. The disease is characterized by a high metastatic rate and a median patient survival of only 4-5 months after diagnosis. Overexpression of ErbB1 has been observed in 62% of NSCLC and contributes to a poor patient outcome. ErbB1 mutations are present in approximately 10% of the NSCLC cases in North America and Western Europe and are usually found in exons encoding the tyrosine kinase domain of the receptor. Such mutations generally cause enhanced kinase activity and subsequent hyperactivation of downstream signaling cascades culminating in increased cell proliferation and survival (Sharma et al., 2007).

The second member of the ErbB family, ErbB2, possesses the most potent mitogenic power as demonstrated by its ability to transform cells even in the absence of a ligand. In fact, ErbB2 is the preferred dimerization partner and heterodimers containing this type of receptor are characterized by an increased mitogenic signaling. This is best exemplified by the ErbB2-ErbB3 heterodimer, which is frequently observed in some of the most aggressive tumors and is associated with poor prognosis (Normanno et al., 2006).

The implication of ErbB2 in tumor formation is best characterized in breast cancer, which is the most common type of cancer in women worldwide. Breast cancer occurs at extremely high incidence, affecting one in nine women in the western world. ErbB2 overexpression is found in approximately 30% of invasive ductal breast carcinomas and correlates with tumor metastasis and shorter survival times. The increased invasive potential of cells overexpressing ErbB2 is partially due to the ability of the receptor to hyperactivate the matrix metalloproteinases MMP-9 and MMP-2, which are involved in degradation of the extracellular matrix. In addition, overexpression of ErbB2 confers resistance to apoptotic signals, which in turn causes decreased sensitivity of cancer cells to various treatments including chemotherapy and hormone therapy (Yu & Hung, 2000).

Tyrosine Kinase Inhibitors and Their Role in Cancer Therapy

The central role of aberrant ErbB signaling in the development and progression of tumors has made the ErbB network an attractive target for pharmacological intervention. Inhibition of ErbB signaling in cancer cells has been intensely pursued over the past two decades and, subsequently, led to the development of targeted cancer therapies, in which

tyrosine kinase inhibitors are used to specifically block the activity of ErbB receptors. Unlike traditional cancer treatments, targeted therapies exhibit a high specificity toward malignant cells, which greatly reduces their toxicity to normal cells. Conventional chemotherapeutic agents do not discriminate between rapidly dividing normal cells and tumor cells which results in multiple toxic side effects. In addition, patients with advanced stages of cancer often exhibit only partial and brief improvement after chemotherapy. Thus, ErbB targeted therapies provide a novel approach to cancer treatment and have already been associated with positive clinical results. Furthermore, targeted therapies can be used in combination with conventional chemotherapy and radiation therapy to produce additive or synergistic effects and further optimize patients' responses (Arora & Scholar, 2005).

ErbB targeted therapies can be broadly divided into two categories: anti-receptor monoclonal antibodies and low-molecular-weight inhibitors of tyrosine kinases. Monoclonal antibodies target the extracellular region of the ErbB receptor and prevent ligand binding or receptor dimerization. In addition, antibodies induce receptor endocytosis leading to ErbB degradation. The resulting inhibition dowregulates proliferation and survival pathways initiated by the ErbB receptors. In addition, mAb can recruit natural killer cells to tumors, which further inhibits tumor growth. A number of monoclonal antibodies targeting the extracellular region of the ErbB receptors have been developed (Table 2) (Bacus et al., 2005).

Table 2: Monoclonal antibodies targeting the ErbB family (Bacus et al., 2005).

Agent	Target	Tumor Type
Cetuximab	ErbB1	Colon, head and neck, lung, pancreas
Panitumumab	ErbB1	Colon, renal
Matuzumab	ErbB1	Head and neck, ovarian, colon, cervix
h-R3	ErbB1	Head and neck
Pertuzumab	ErbB2	Breast, ovarian, prostate, lung
Trastuzumab (Herceptin)	ErbB2	Breast

Two of the ErbB monoclonal antibodies, Cetuximab and Herceptin, have been approved by the Food and Drug Administration and are currently used in the treatment of lung and

breast cancer, respectively. Clinical data indicate that patients with metastatic breast cancer had an improved overall survival when treated with Herceptin and chemotherapy as opposed to chemotherapy alone (Slamon et al., 2001). Similarly, clinical trials with Cetuximab showed a prolonged overall survival in patients with advanced non-small cell lung carcinoma who received a combination of Cetuximab and chemotherapy (Pirker et al., 2009).

In contrast to mAb, which bind to the extracellular portion of the ErbBs, low-molecular-weight inhibitors target the intracellular kinase domain and lead to inhibition of the kinase activity and subsequent abrogation of receptor signaling. Small molecule tyrosine kinase inhibitors can be further divided into reversible and irreversible inhibitors. Reversible small molecule inhibitors bind at the vicinity of the ATP-binding pocket of their target kinases, mimicking ATP binding. Thus, these inhibitors compete with the endogenous ATP for binding to the kinase domain. Irreversible inhibitors usually react with a cysteine residue at the kinase ATP pocket to form a covalent bond. As a result, the inhibitor irreversibly blocks ATP binding to the kinase domain and completely abolishes kinase activity (Madhusudan & Ganesan, 2004).

Several small molecule tyrosine kinase inhibitors targeting the ErbB receptors have been developed and approved by the FDA for treatment of cancer. Some of the most promising and extensively characterized drugs include gefinitib and erlotinib for the treatment of non-small-cell lung carcinoma, and lapatinib for the treatment of breast cancer (Table 3).

Table 3: Small molecule tyrosine kinase inhibitors targeting the ErbB family (Bacus et al., 2005).

Agent	Reversible	Target	Tumor Type
Gefitinib	Yes	ErbB1	Lung
Erlotinib	Yes	ErbB1	Lung, pancreas
Lapatinib	Yes	ErbB1/2	Breast
CI-1033	No	Pan ErbB	Squamous cell carcinoma, skin
EKB-569	No	ErbB1	Colon

Gefitinib and erlotinib are reversible inhibitors of ErbB1 kinase domain and *in vitro* studies with cancer cell lines demonstrated that dose-depended inhibition of ErbB1

autophosphorylation was associated with decreased cell growth. Clinical trials subsequently showed that gefitinib improve survival rates only in a subgroup of patients who have specific activating ErbB1 mutations. These mutations are associated with enhanced receptor signaling and conferred increased receptor susceptibility to gefinitib. On the other hand, erlotinib has been demonstrated to significantly improve survival rates of paients overexpressing wild type ErbB1 (Laack et al., 2010).

Lapatinib is a dual specificity inhibitor and was approved by the FDA for the treatment of advanced or metastatic breast cancer in combination with other therapies. Results from clinical studies indicate that the drug is beneficial to patients and in combination with chemotherapy can increase time to disease progression and progression-free survival rates (Paul et al., 2008).

As a class of molecularly targeted therapies, small molecule tyrosine kinase inhibitors have made a substantial contribution towards improvements in cancer therapies. They are usually well tolerated, with more manageable toxicity profiles as compared to conventional cytotoxic chemotherapies. In addition, small molecule tyrosine kinase inhibitors represent a novel approach for the treatment of patients with advanced stages of cancer and have improved the quality of life of patients with poor prognosis. Unfortunately, patients treated with mAb or small molecule tyrosine kinase inhibitors eventually develop drug resistance. Selective pressure in the rapidly dividing tumor cells leads to emergence of drug-resistant ErbB receptor variants with kinase domain mutations that abrogate drug binding to the ATP-pocket. Therefore, there is a need to develop novel inhibitors that circumvent the resistance problem. One approach is to design drugs that can tolerate a range of amino acids at the gatekeeper position of the kinase catalytic pocket. The gatekeeper residue determines the accessibility of a hydrophobic pocket located next to the ATP-binding site. Hydrophobic interactions between the receptor and the inhibitor in the hydrophobic pocket are essential for the inhibitor binding affinity. A second strategy to overcome drug resistance is to target the kinase domain with inhibitors binding at alternative sites (Zhang et al., 2009).

The need to discover novel tyrosine kinase inhibitors comes with the challenge to evaluate drug potency and selectivity at the organismal level. *In vitro* studies with cell culture demonstrate that different cancer cell lines often exhibit markedly different responses to the same inhibitor. Therefore, new approaches to evaluate a given inhibitor in the context of a living organism may provide a more accurate assessment of the drug potency and efficacy (Zhang et al., 2009).

Drosophila as a Model System for Drug Validation

Despite rapid advances in elucidating the molecular basis of cancer, oncology drug discovery remains a challenging undertaking. Anti-cancer drug candidates have significantly lower rates in clinical development compared to compounds in other areas such as cardiovascular and infectious diseases (Kamb et al., 2006). A crucial step in the process of developing a therapeutic agent is early validation of the efficacy of drug candidates. Cultured cancer cell lines are widely utilized in the early stages of drug discovery to screen libraries of compounds for an ability to produce a desired physiological outcome. Although conventional cell culture assays are convenient to use in a high-throughput screen, they have several major limitations. Cell culture systems cannot recapitulate many aspects of the *in vivo* cell microenvironment and, therefore, fail to account for many of the complex cell and tissue interactions that occur at the level of an intact organism. As a result, many drug candidates selected on the basis of cell culture assays fail to exhibit the necessary level of efficacy in subsequent validation stages in animal models. To overcome these limitations, novel drug validation approaches need to be incorporated into the early stages of the drug discovery process (Bell et al., 2009).

The sequencing of human and other eukaryotic genomes has revealed a striking conservation of biochemical modules that control fundamental cellular processes such as proliferation, differentiation and migration. It is, therefore, feasible to consider utilizing invertebrate model organisms as a drug discovery and validation tool to increase the rate of discovery of higher quality leads.

The fruit fly *Drosophila melanogaster* possesses several compelling features that make it a particularly attractive model organism to use for *in vivo* drug validation. *Drosophila* has a short generation time and large populations can be grown in a laboratory setting at an extremely low cost, which greatly enhances the efficiency of the experimental studies. This is coupled with sophisticated tools for genetic manipulation, as well as a complete genome sequence (Matthews & Kopczynski, 2001). In fact, comparative genome analyses indicate that 61% of the disease genes in humans have been conserved in flies. Moreover, many vital developmental pathways and cascades are also conserved from flies to humans. Thus, it is possible to use *Drosophila* as a simple organism to dissect the molecular basis of complex diseases such as cancer. For instance, a study of the oncogene *ras* in flies led to the identification of novel genes that modulate the same signaling network in humans. In addition, the guanine nucleotide exchange factor SOS (son-of-sevenless), which promotes exchange of GDP to GTP on Ras and activates it was first identified in *Drosophila* (Muda & McKenna, 2004).

Perhaps one of the most compelling features of *Drosophila* as a model system is the ability to target gene expression in a temporal and spatial manner using the driver-responder GAL4/UAS system. In this binary system, a gene of interest is cloned behind a UAS (upstream activating sequence) element, which contains five GAL4 binding sites. In the absence of the transcription factor GAL4, the gene of interest (responder) is transcriptionally silent. When flies containing the responder are crossed to lines expressing GAL4 in a tissue-specific manner, the resulting progeny expresses the gene of interest as a result of GAL4 binding to the UAS. This elegant tool for genetic manipulation has been invaluable for investigating gene function *in vivo* (Duffy, 2002).

Due to its small size, short generation time and low cost of culture, *Drosophila* can also be used in high-throughput screens of libraries of compounds. In fact, whole animal assays can be set up on standard microtiter plates (96 wells) to identify or validate novel anti-cancer drugs or elucidate the mode of action of a given compound. Such high-throughput *in vivo* pharmacological screens have low costs and can increase the efficiency of the drug discovery and validation process (Muda & McKenna, 2004).

The high degree of conservation of the ErbB signaling module between *Drosophila* and humans makes it feasible to consider flies as potential model organism for *in vivo* pharmacological assays related to the ErbB receptors.

Epidermal Growth Factor Receptor Signaling in Drosophila Oogenesis

In *Drosophila* the epidermal growth factor receptor (DER) specifies cell fates and has several important roles throughout development. During oogenesis the receptor is required for establishment of the dorsoventral and anterior-posterior axes of both the eggshell and the embryo (Shilo, 2003).

Drosophila female flies have two ovaries containing approximately 18 ovarioles. A specialized structure at the tip of each ovariole, the germarium, gives rise to egg chambers which mature into eggs as they pass down the ovariole. Each egg chamber consists of a germline-derived oocyte and nurse cells enclosed in a monolayer of follicular epithelium (Fig. 6) (Cavaliere et al., 2008). In the early stages of oogenesis DER signaling in the posterior follicle cells establishes the anterior-posterior axis of the egg chamber. In the later stages of oogenesis, DER signaling is confined to a subpopulation of dorsal-anterior follicle cells, where the receptor specifies the dorsal fate of these cells and establishes the dorsoventral polarity of the eggshell. DER signaling in the follicle cells is activated by a TGF-α-like ligand, Gurken, which is expressed on the surface of the oocyte and only proximal to the nucleus. During oogenesis, the nucleus moves from its initial position at the posterior end of the oocyte to the dorsal anterior site, thus causing a re-localization of Gurken expression. DER signaling also specifies the differentiation and positioning of the two dorsal-anterior appendages in the embryo. Inhibition of DER signaling causes ventralization of the chorion and loss of the two dorsal appendages. On the other hand, overexpression of the receptor leads to dorsalization of the chorion (i.e. ectopic expression of dorsal appendages) (Wasserman & Freeman, 1998) .

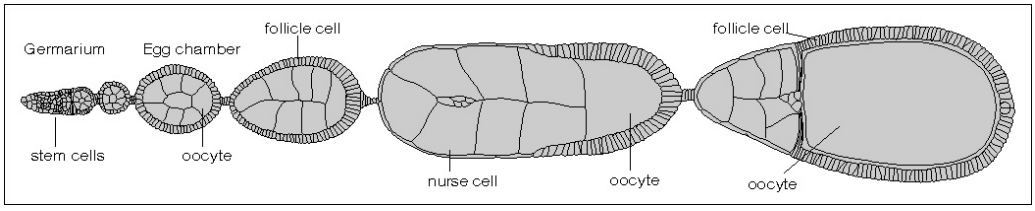


Figure 6: Stages of *Drosophila* Oogenesis. (University of Newfoundland, 2010)

Materials and Methods

Generating ErbB Expression Clones

The Gateway Cloning System was used to generate ErbB expression clones. The Gateway Cloning Technology is a universal cloning system in which DNA fragments can be cloned into a variety of vectors without the use of restriction endonucleases. Instead, the Gateway Technology takes advantage of the lambda phage recombination system, which is used to transfer DNA fragments into vectors containing specific recombination sites called *att* sites.

Two types of in vitro reactions, BP and LR, are mediated by a mix of clonase enzymes and transfer a DNA sequence of interest first into an entry vector and then into a final expression vector. The BP reaction is a recombination between a gene of interest flanked by *attB1* and *attB2* sites, and a donor vector containing a ccdB gene flanked by *attP1* and *attP2* sites. In the resulting entry clone, the DNA sequence of interest is flanked by *attL* sites. The LR reaction is then a recombination between the *attL* sites of the donor vector and the *attR* sites of the destination vector. The resulting expression clone contains the gene of interest flanked by *attB* sites and can be used to generate transgenics.

Selection of positive clones is achieved by the presence of a kanamycin and ampicillin resistance genes in the Gateway vectors. In addition, empty entry and destination vectors contain a ccdB gene, which is toxic to *E. coli* and prevents growth on medium. Thus, only clones containing the gene of interest are recovered, which greatly increases the efficiency of the cloning process (Fig. 7) (Invitrogen).

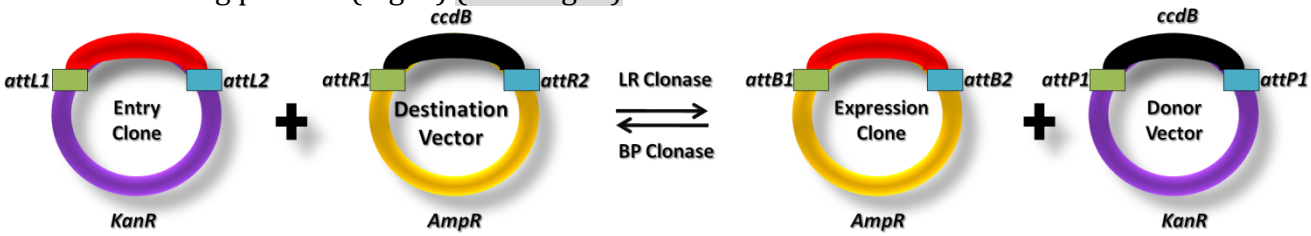


Figure 7: Gateway Recombination Cloning Technology (Adapted from Ivitrogen)

ErbB2 entry clones previously generated in the Duffy Lab were used in an LR reaction with a pUAST GFP destination vector to create an expression clone: 7µL pENTR ErbB2, 1µL of 150ng/µL pUAST GFP and 2µL LR Clonase mix were incubated at 25^{0}C overnight. 5µL of

the LR reaction were transformed into DH5α Max Cloning Efficiency cells and plated on LB agar plates containing 50μg/mL ampicillin. Colonies were grown overnight in small liquid LB cultures containing ampicillin and were then miniprepped using Qiagen's Qiaprep Spin Miniprep Kit. Positive clones were verified by restriction enzyme digestion and midiprepped with Qiagen's Plasmid Midi Kit. The DNA was then sent to Yale's DNA Analysis Facility for sequencing and the resulting chromatograms were analyzed using the Sequencher software.

Generation and Mapping of ErbB1 and ErbB2 Transgenic *Drosophila*

The midiprepped DNA for pUAST-ErbB2-GFP expression clone and maxiprepped DNA for pUAST-ErbB1-GFP (previously generated in the Duffy Lab) was sent to Genetic Services, Inc. (Cambridge, MA) for injection into w^{1118} (wild type) *Drosophila* embryos. The resulting surviving larvae were returned to the lab for transgenic screening and mapping. Surviving flies (generation G_0) were single-pair mated to 3-4 w^{1118} males or virgin females depending on the sex of the putative transgenic. w^{1118} flies have no eye pigmentation but the expression constructs injected into embryos contained the *white* gene responsible for eye color production. Therefore, eye-colored progeny from the previous mating indicated presence of the transgene. These transgenics (generation F1) were collected and mated to flies of the genotypes [w^-; Sp/CyO (chromosome II); +/+] and [w^-; +/+; Ly/TM3 Sb (chromosome III), for mapping purposes. From these matings, heterozygous progeny containing both eye color and the Balancer chromosome marker (CyO or TM3 Sb) were collected and outcrossed back to w^{1118} flies to determine the segregation pattern of the transgene versus the marker. Figure 8 and 9 depict the mapping scheme of transgenic strains containing an insert in the second and third chromosomes, respectively.

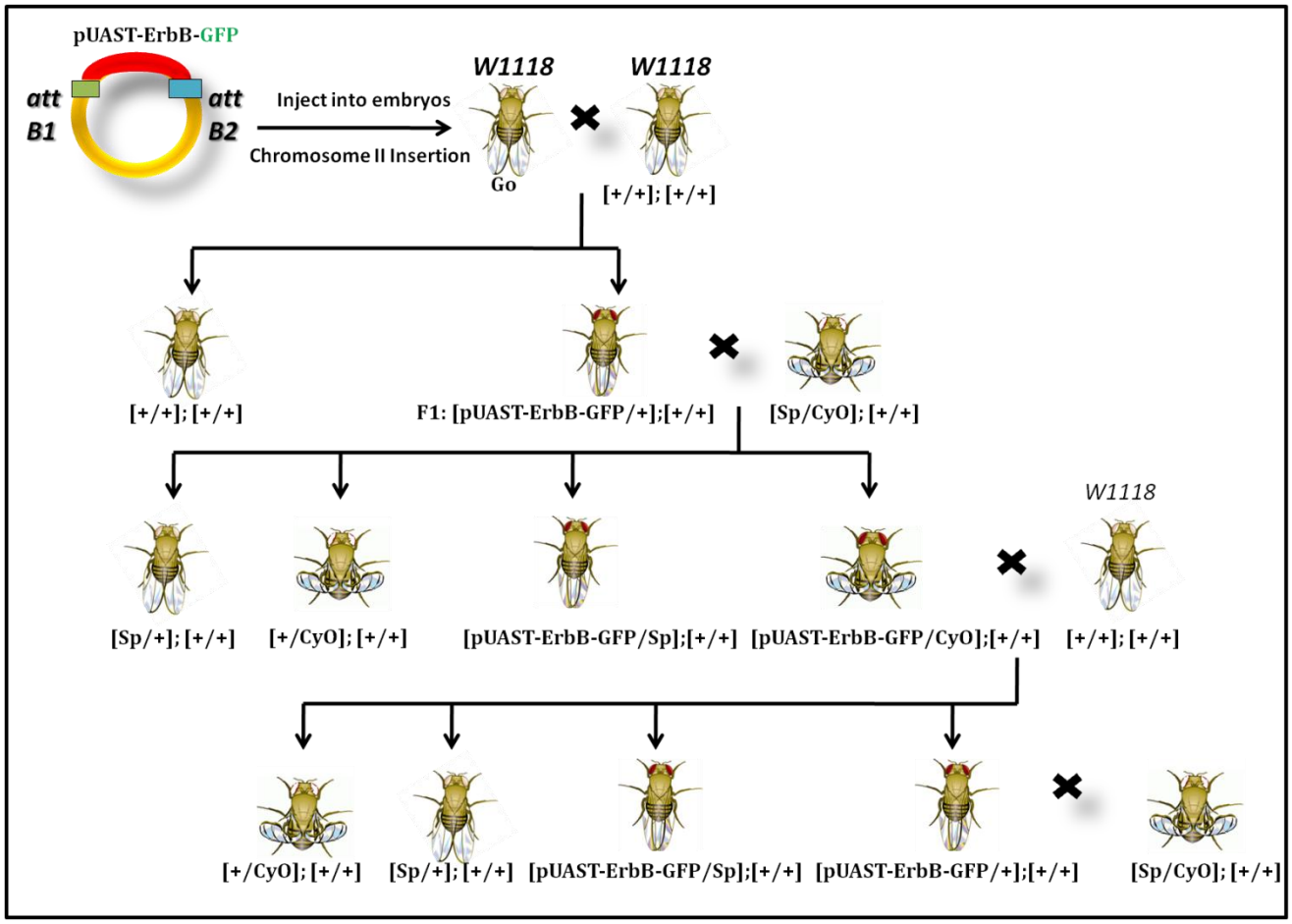


Figure 8: Transgene Mapping Outline (Chromosome II insertion)

A consistent segregation of the transgene away from the Balancer chromosome marker indicates transgene insertion into that respective chromosome. For instance, if the transgene always segregates away from the CyO marker, the construct must have inserted into the second chromosome.

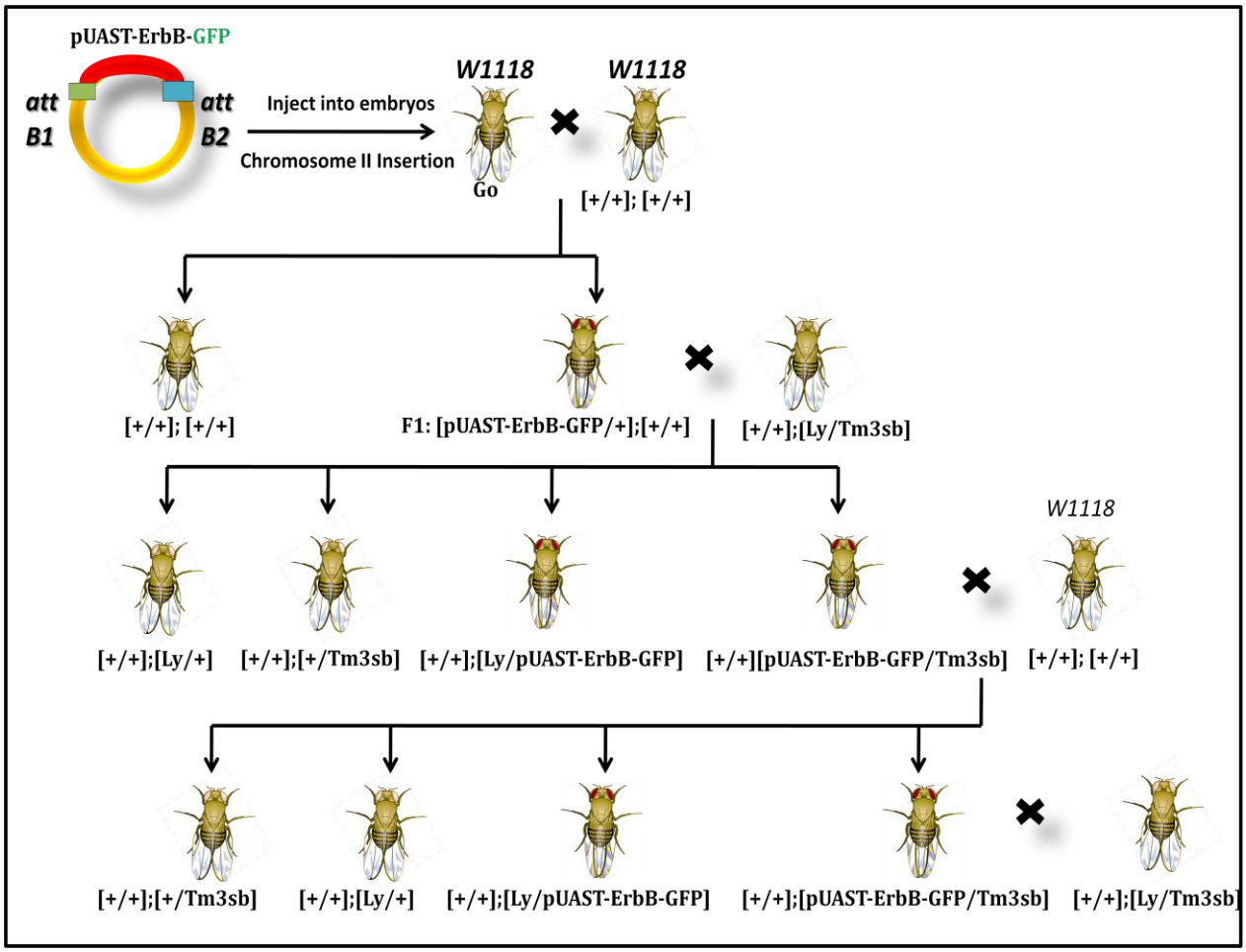


Figure 9: Transgene Mapping Outline (Chromosome III insertion)

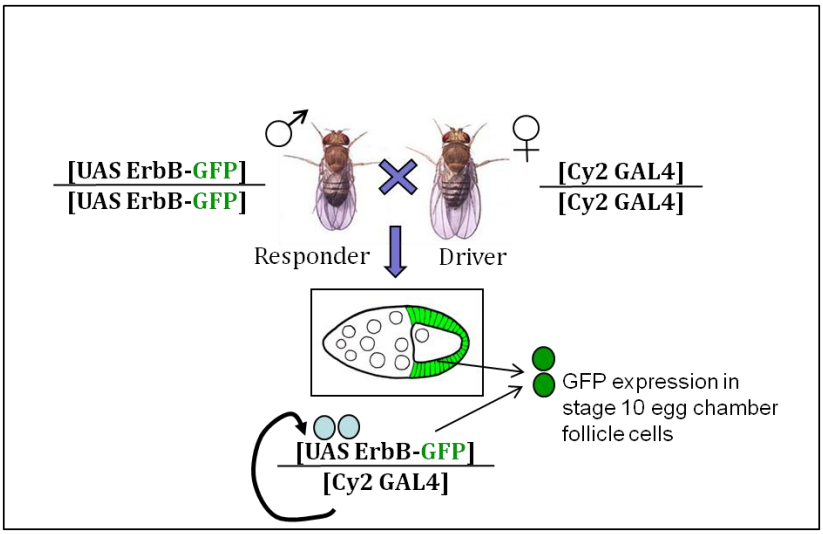
Once mapped, stable stocks of the transgenic strains were generated by first mating one transgenic male of the genotype [pUAST-ErbB-GFP/+] to 4-5 males of the respective balancing strain [Sp/CyO for chromosome II insertion or Ly/TM3 Sb for chromosome III insertion]. From this cross, female and male progeny of the genotype [pUAST-ErbB-GFP/CyO or pUAST-ErbB-GFP/TM3 Sb] were mated to each other to generate the final stable transgenic stock.

For transgene insertions into the X chromosome (chromosome I), transgenic females were mated to *FM6, y w B* males. From this mating, female progeny of the genotype [pUAST-ErbB-GFP/ *FM6, y w B*] were crossed back to *FM6, y w B* males to generate the final stable transgenic stock.

Gain-of-function Studies with the Gal4/UAS System

The Gal4/UAS system is used for targeting gene expression in a temporal and spatial manner by mating a driver strain (GAL4) to a responder strain (UAS). In this binary system, a gene of interest is cloned behind a UAS (upstream activating sequence) element, which contains five GAL4 binding sites. In the absence of the transcription factor GAL4, the gene of interest (responder) is transcriptionally silent. When flies containing the responder are crossed to lines expressing GAL4 in a tissue-specific manner, the resulting progeny expresses the gene of interest as a result of GAL4 binding to the UAS. Expression levels of the responder gene are also dependent on the temperature at which the cross is carried out. Higher temperature corresponds to higher expression levels (Duffy, 2002).

The CY2 GAL4 driver was used in these studies to misexpress ErbB1, ErbB2 and DER during oogenesis(Fig.10) Female CY2 GAL4 driver flies were crosses to 5-6 [pUAST-ErbB1/ErbB2/DER-GFP] males. For strains containing transgene insertion in the X chromosome, male CY2 GAL4 driver flies were mated to responder (UAS) females. All crosses were set up and maintained at 20^0C



Figre 10: ErbB Missexpression during Oogenesis

Adult Ovary Dissection and Mounting

Adult female ovaries were dissected in PBT, fixed in 3.7% formaldehyde for 15 min and mounted on a slide in 50% glycerol in PBP. GFP localization in stage 10 egg chambers was

examined using a fluorescent Zeiss Imager.Z microscope with Apotome at 20X. Images were captured using a Zeiss Axiocam and were processed with Zeiss's Axiovision software.

Chorion Preps

Female and male flies overexpressing ErbB1, ErbB2 or DER during oogenesis were placed on apple juice egg lay plates covered with a small beaker. The flies were kept at 25^0C overnight. On the following day, the original plates were replaced with new ones and chorions were collected and mounted on a microscope slide in Lacto-Hoyer's (1:1) solution. The slide was placed on a hotplate overnight at 65^0C. Chorions were examined for a dorsalization phenotype usinhg dark field microscopy on a Zeiss Imager.Z1 (10X).

Drug Screening Assay Design

The drug treatment experiments were set up in an 80-well eppie rack. Each well contained 1mL apple juice agar prepared by the following recipe: 3.3g agar were added to 60mL of dH_2O and 90mL apple juice in a 1L plastic beaker. The mixture was microwaved for 1.5 min after which 5g sugar were added. 1mL of the mixture was poured into each well and left to solidify.

The small molecule tyrosine kinase inhibitors CL-387,785 and PD 168393 ordered from Calbiochem were dissolved in 200µL DMSO to yield a concentration of approximately 13.5mM. Apple juice and dH_2O mixed in a 1:1 ratio were used to dilute the CL-387,785 (CL) and PD 168393 (PD) stocks to 1:100. 10µL of each inhibitor dilution were pipetted on top the solidified apple juice agar in the wells. Female flies overexpressing ErbB1 or ErbB2 during oogenesis were then placed in the wells together with two male flies. The wells were covered with scotch tape with small holes to allow in air. The racks were kept at 25^0C. After 24 hours the flies were transferred to new racks containing freshly poured apple juice agar and the same amount and concentration of inhibitor solution. The overall drug screening set up is outlined in Fig. 11.

The chorion phenotype was scored every 24 hours over the course of 7 days. For each transgenic strain, the inhibitor treatment was done in triplets and also included an untreated control.

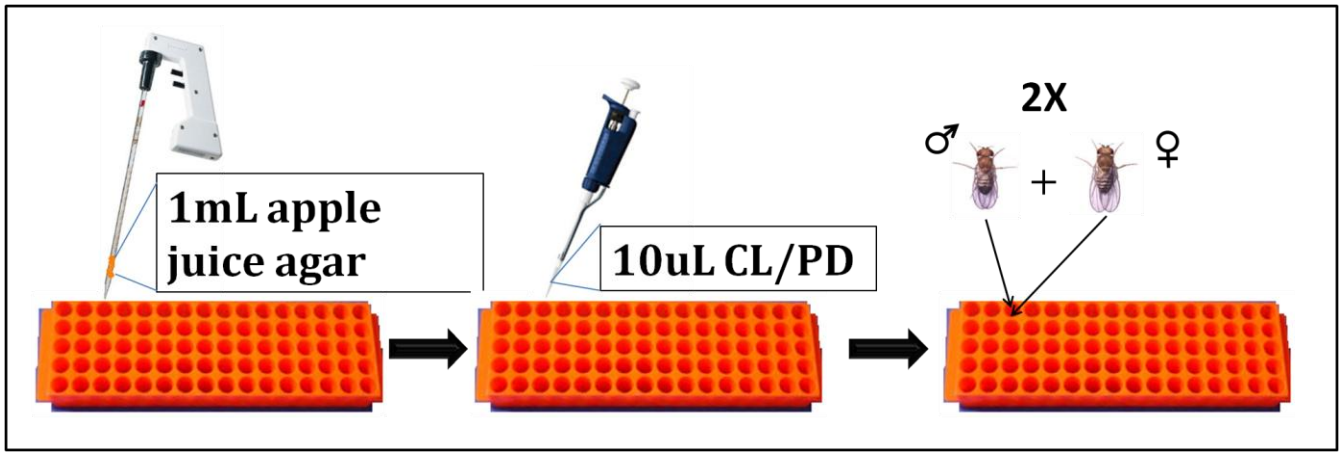


Figure 11: Drug Screening Assay Set Up

Results

Transgenic *Drosophila* strains containing the human ErbB1 or ErbB2 receptors were successfully generated and mapped. To characterize receptor localization and activity, ErbB1 and ErbB2 were missexpressed during *Drosophila* oogenesis. These functional tests demonstrated that both receptors have activity and are properly localized. Furthermore, in some transgenic strains tyrosine kinase inhibitor treatments reversed the phenotype caused by missesxpression of the human receptors.

Transgenic Drosophila Strains Mapping

Constructs pUAST-ErbB1-GFP and pUAST-ErbB2-GFP were sent to Genetic Services, Inc. for injection into embryos. Surviving larvae were returned to the lab and the resulting adults were single-pair mated to w^{1118} flies of the opposite sex. The transgenic progeny were selected based on eye color and were again single-pair mated to w^{1118} flies to map the position of the transgene as outlined in Materials and Methods. For each construct, 13 transgenic strains were obtained and mapped (Table 4&5).

Table 4: pUAST-ErbB1-GFP Mapping Results

Strain	Color	Chromosome
SAA3M-1F	Dark Orange	3
SAA4M-1M	Orange	3
SAA3F-1M	Dark Orange	2
SAA6F-1M	Orange	1
SAA7F-1M	Red	3
SAA9M-1M	Orange	3
SAA11F-1M	Red	2
SAA15M-1M	Dark Orange	2
SAA17M-1F	Yellow with patches of red	2
SAB2M-1M	Orange	3
SAB8M-1M	Dark Orange	3
SAB9F-1M	Red	2
SAB10F-1M	Red	3

Table 5: pUAST-ErbB2-GFP Mapping Results

Strain	Color	Chromosome
ARA8M-1M	Orange	2
ARA13F-1M	Orange	1
ARA15F-1M	Dark Orange	2
ARA16F-1M	Dark Orange	2
ARA24F-1M	Dark Orange	3
ARA26F-1M	Dark Orange	3
ARA28F-1M	Orange	3
ARA36M-1M	Dark Orange	3
ARA37M-1M	Orange	3
ARA38M-1M	Dark Orange	3
ARA43M-1M	Orange	2
ARA51M-1M	Dark Orange	3
ARA53M-1M	Orange	2

Functional Analysis of Human ErbB Receptors in *Drosophila*

To characterize the localization and activity of the human receptors in *Drosophila,* ErbB1 and ErbB2 were missexpressed by crossing transgenic strains to CY2 GAL4 flies as outlined in Materials and Methods. In the larval stages, CY2 GAL4 causes expression in the salivary glands. During oogenesis CY2 drives protein expression in the follicle cells surrounding egg chambers. Localized DER signaling specifies the dorsal fates of a subpopulation of follicle cells and establishes the dorsoventral axis of the embryo. Previous studies have shown that misexpression of DER during oogenesis results in dorsalization of the chorion. This phenotype is also characterized by fusion and ectopic expression of the dorsal respiratory appendages of the embryo (Queenan et al., 1997). Thus, the dorsalized chorion phenotype was used in these functional studies as a measure of receptor activity.

Since preliminary functional analysis indicated that missexpression of the transgenes causes lethality at room temperature, the cross between the CY2 GAL4 driver and the transgenic responder strain was set up and maintained at 20^0C. The developing larvae and pupae were examined for GFP expression in the salivary glands. The F1 progeny were then collected and transferred to 25^0C to examine chorions for a dorsalization phenotype. GFP expression in the larval and pupal salivary glands was indicative of full translation of the ErbB1 and ErbB2 receptors, while the dorsalized chorion phenotype indicated receptor activity. The results are summarized in tables 6 and 7.

Five ErbB1 strains (SAA4M-1M, SAA7F-1M, SAA11F-1M, SAB8M-1M, SAB10F-1M) exhibited strong GFP expression in larval and pupal stages and correlated with a consistent dorsalized chorion phenotype at 25^0C. Except for SAB8M-1M, these strains had relatively high viability (Table 6).

Of the 13 ErbB2 transgenic strains, 5 were lethal even at 20^0C. Only one strain, ARA15F-1M, exhibited the dorsalized chorion phenotype at 25^0C (Table 7).

Table 6: CY2 Gal4 Misexpression of pUAST-ErbB1-GFP.

Strain	GFP in Salivary Glands (20^{0}C)		Chorion Phenotype (25^{0}C)
	Larva	Pupa	
SAA3M-1F	+	+	WT
SAA4M-1M	**+**	**++**	**Dorsalized**
SAA3F-1M	+	+	WT
SAA6F-1M	+	+	WT+Dorsalized
SAA7F-1M	**+++**	**+++**	**Dorsalized**
SAA9M-1M	-	-	WT
SAA11F-1M	**+++**	**+++**	**Dorsolized**
SAA15M-1M	+++	+++	**WT+Dorsalized**
SAA17M-1F	+++	+++	WT+Dorsalized
SAB2M-1M	+	+	WT
SAB8M-1M	**+++**	**+++**	**Dorsalized**
SAB9F-1M	+	+	WT
SAB10F-1M	**+++**	**+++**	**Dorsalized**

Table 7: Cy2 Gal4 Misexpression of pUAST-ErbB2-GFP.

Strain	GFP in Salivary Glands (20^{0}C)		Chorion Phenotype (25^{0}C)
	Larva	Pupa	
ARA8M-1M	-	-	WT
ARA13F-1M	+	lethal	
ARA15F-1M	**+**	**+**	**Dorsalized**
ARA16F-1M	-	-	WT
ARA24F-1M	+	lethal	
ARA26F-1M	-	-	WT
ARA28F-1M	-	+	WT
ARA36M-1M	lethal		
ARA37M-1M	+++	+++ only in prepupa	WT
ARA38M-1M	lethal		
ARA43M-1M	+++	lethal	
ARA51M-1M	+	++	WT
ARA53M-1M	+	++	WT

To characterize the pattern of localization of the human receptors, ovaries from female flies overexpressing ErbB1 or ErbB2 were dissected and the follicular epithelium of stage 10 egg chambers was examined for GFP expression. As shown in Fig. 12A, the follicle cells

form a monolayer that surrounds the egg chamber. To determine the overall level of receptor activity, chorions were collected and examined for the degree of dorsalization. As shown in Fig. 12B, a wild type chorion has a well-defined dorsal-ventral axis with two appendages on the dorsal side. A dorsalized chorion is characterized by ectopic expression of dorsal appendages.

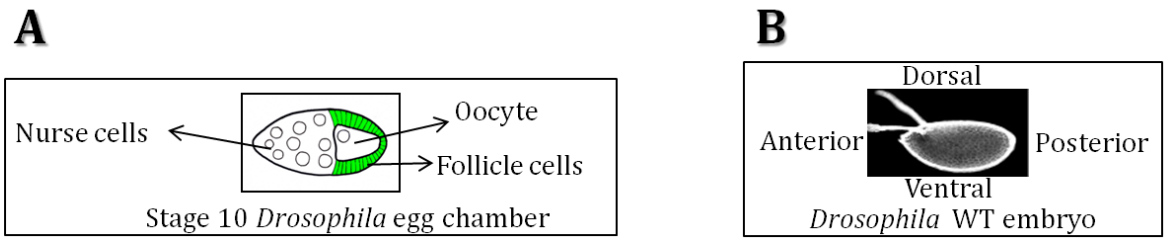


Figure 12: A, Stage 10 Drosophila egg chamber; B, Wild type *Drosophila* chorion.

The *Drosophila* EGFR receptor (DER) was also missexpressed during oogenesis with the CY2 GAL4 driver. DER was used as a control and was compared to human receptors. ErbB1 and ErbB2 were expressed within the follicle cells of stage 10 egg chambers and their expression was properly localized to the cell membrane (Fig. 13A). The levels of expression were relatively similar across the ErbB1 and ErbB2 transgenic strains. However, the DER strain exhibited a lower expression level and a different localization pattern. Although the receptor was also properly localized to the cell membrane, not all follicle cells expressed the protein at the same level (Fig. 13A). It is possible that the lower expression levels of DER were due to receptor endocytosis and subsequent lysosomal degradation through recruitment of the Cbl ubiquitin ligase. The *Drosophila* Cbl may not recognize the phosphotyrosine residues at the C-terminal tail of the human receptors as binding sites. As a result, once endocytosed, ErbB1 and ErbB2 would not be ubiquitinated by Cbl. Therefore, rather than degraded, the human receptors are probably recycled back to the cell membrane.

Misexpression of both the human receptors and DER during oogenesis resulted in a dorsalized chorion phenotype (Fig. 13B), suggesting that ErbB1 and ErbB2 were active. Chorions from strains overexpressing DER had a less severe and consistent dorsalized

phenotype as compared to the transgenic ErbB1 and ErbB2 strains. In particular, ErbB1 strain SAA7F-1M, and ErbB2 strain ARA15F-1M, exhibited a severe dorsalized phenotype as demonstrated by a characteristic peanut chorion shape (Fig. 13B).

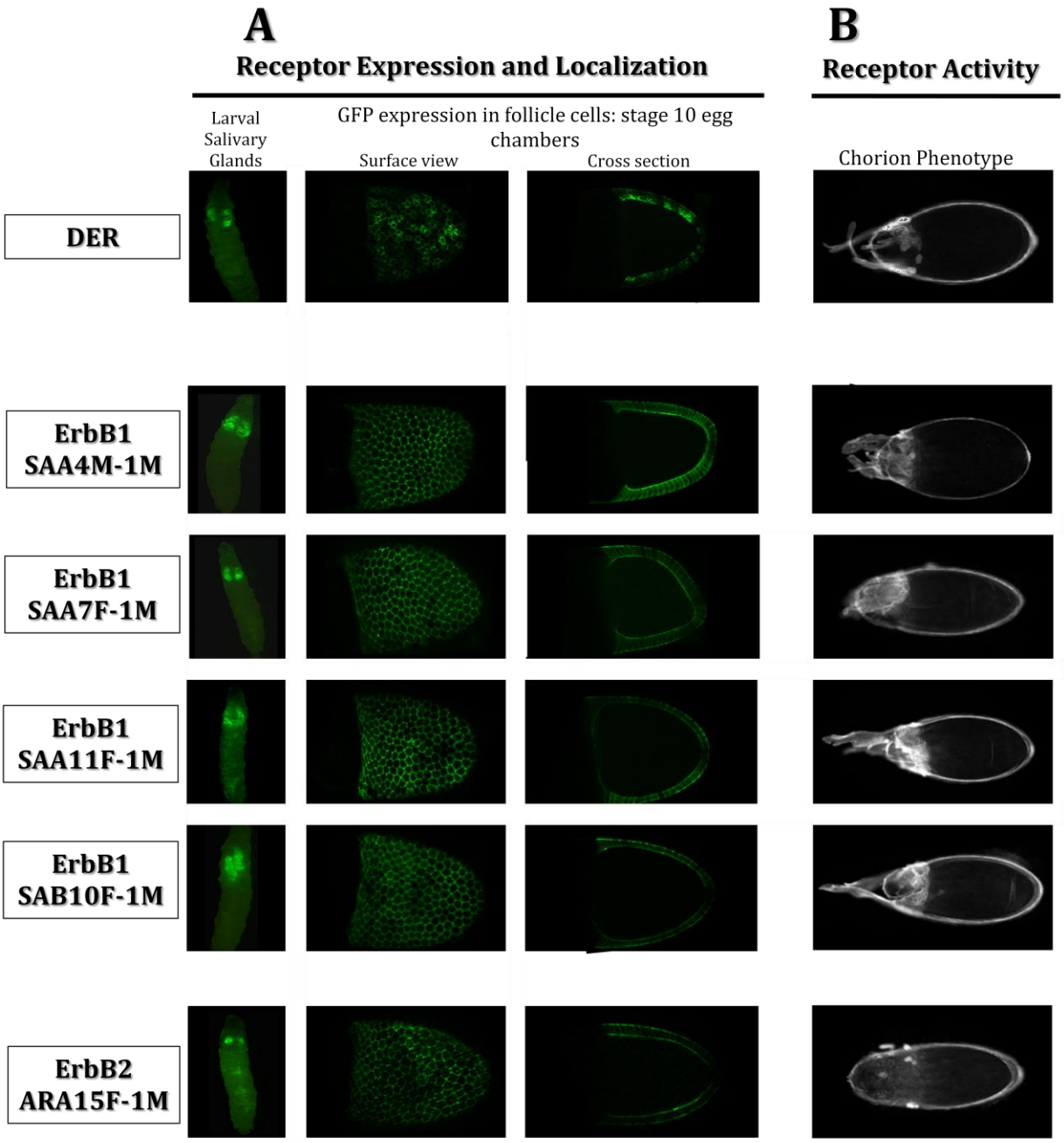


Figure 13: A, Receptor expression levels and localization pattern; B, chorion phenotypes induces by receptor misexpression

Tyrosine Kinase Inhibitor Screening Assay

The transgenic ErbB *Drosophila* strains that exhibited proper receptor localization and receptor activity demonstrated by a dorsalized chorion phenotype were used in a drug screening assay as described in Materials and Methods. During oogenesis DER specifies the dorsal fates of the egg chamber follicle cells and, thus, establishes the dorsoventral axis of the developing egg chamber, chorion and embryo. DER signaling also specifies the differentiation and positioning of the two dorsal-anterior appendages in the chorion. Inhibition of DER signaling causes ventralization of the chorion and loss of the two dorsal appendages. On the other hand, overexpression of the receptor leads to chorion dorsalization and ectopic expression of dorsal appendages (Wasserman & Freeman, 1998).

Previous work in the Duffy Lab by C. Leduc demonstrated that feeding transgenic flies overexpressing ErbB1 with small molecule tyrosine kinase inhibitors resulted in reversion of the dorsalized chorion phenotype to a wild type phenotype. This provides a simple way to assess the activity and potency of novel small molecule tyrosine kinase inhibitors and can potentially be adopted as a whole animal in vivo approach to identify or validate novel cancer therapeutics.

Two known tyrosine kinase inhibitors, CL-387,783 and PD 163393 (Fig. 14), were tested for an ability to suppress the dorsalized chorion phenotype in the transgenic strains overexpressing ErbB1 and ErbB2. CL-387,783 (N-[4-[(3-Bromophenyl)amino]-6-quinazolinyl]-2-butynamide) and PD 163393 (4-[(3-Bromophenyl)amino]-6-acrylamidoquinazoline) belong to a quinazoline class of irreversible inhibitors. Experimental data demonstrate that the two TKI inhibit ErbB signaling by covalently binding to a cysteine residue at position 773 in the ATP pocket of the receptor, thus preventing subsequent ATP binding (Discafani & Carroll, 1999; Fry et al., 1998). CL-387,783 was shown to be specific to ErbB1 (Discafani & Carroll, 1999), while experimental data suggest that PD 163393 can bind and inhibit both ErbB1 and ErbB2 (Fry et al., 1998).

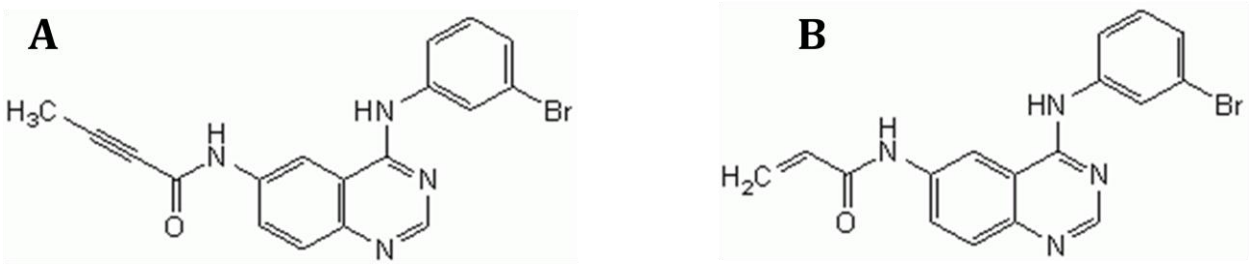


Figure 14: Structure of CL-387,783 (A) and PD 163393 (B) (Taken from EMD/Calbiochem website: http://www.emdbiosciences.com/product/233100 and http://www.emdchemicals.com/life-science-research/products/EMD_BIO-513033/p_uuid)

Four ErbB1 (SAA4M-1M, SAA7F-1M, SAA11F-1M and SAB10F-1M) and one ErbB2 (ARA15F-1M) transgenic strains were used in the drug screening assay. The effect of the inhibitors on wild type flies was also tested.

Functional analysis of the human receptors revealed that two of the ErbB1 transgenic strains, SAA4M-1M and SAA7F-1M, exhibited a strong and very consistent dorsalized chorion phenotype when the receptor was overexpressed during oogenesis. Treatment of these strains with CL-387,783 increased the appearance of wild type chorions from 0% (untreated strains) to 14% in SAA4M-1M and 5% in SAA7F-1M (Fig. 15). In addition, treated flies from the strain SAA7F-1M appeared to lay larger amounts of chorions than the untreated flies. Strains SAA11F-1M and SAB10F-1M also exhibited strong chorion dorsalization but the phenotype was less consistent and the flies were laying a small number of wild type chorions as well. Treatment with the CL-387,783 inhibitor increased the appearance of wild chorions from 16% to 33% in SAA11F-1M and from 8% to 32% in SAB10F-1M. No effect was observed on the wild type strain since both treated and untreated flies laid only wild type chorions (Fig. 15). Although CL-387,783 is not specific for ErbB2, the transgenic line expressing this receptor was still treated with the inhibitor. No effect on the ErbB2 strain (ARA15F-1M) was observed, since both the treated and untreated females laid severely dorsalized eggs (Data not shown).

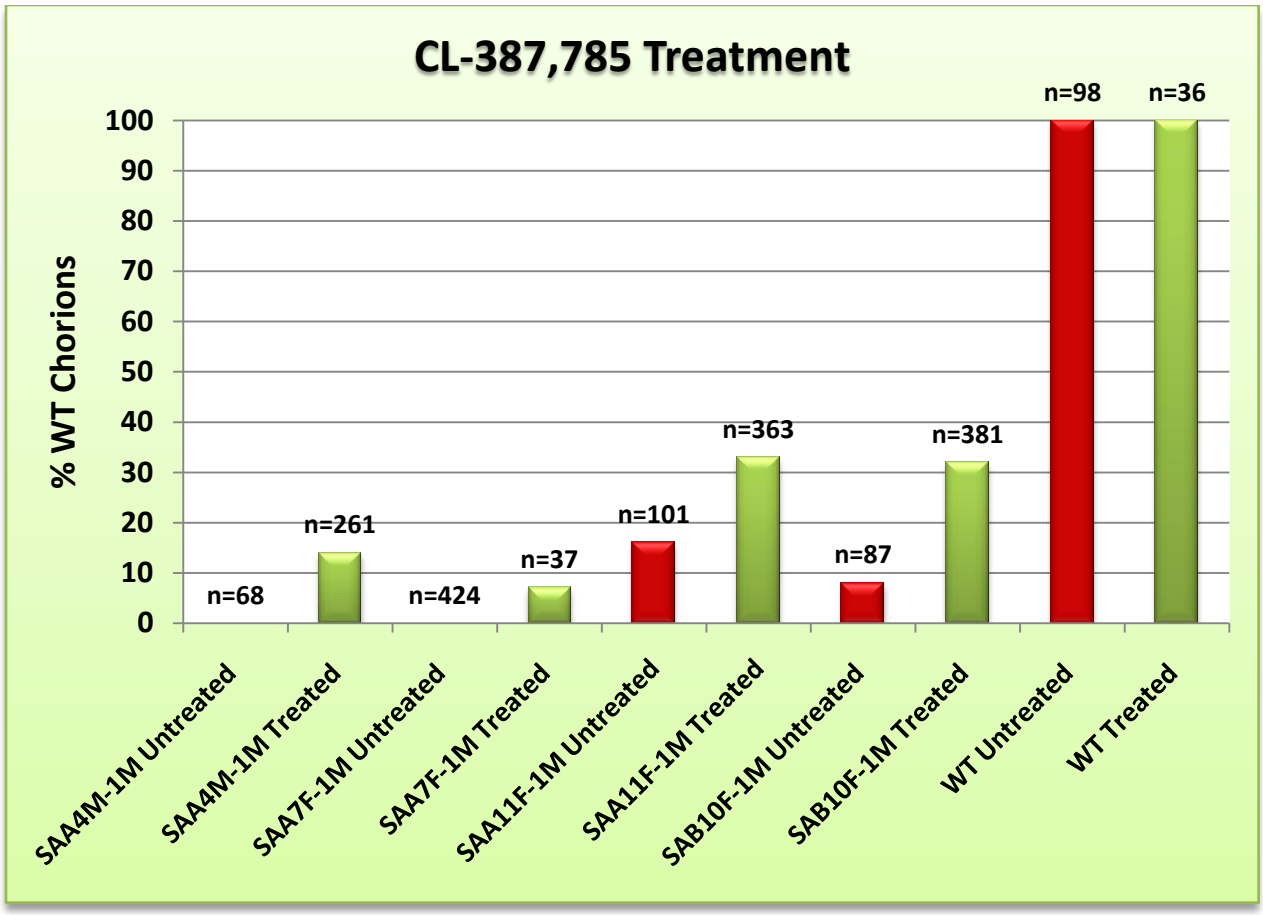


Figure 15: Effect of CL-387,783 (1:100 dilution) treatment on ErbB1 and wild type *Drosophila* strains (n=total number of chorions)

Treatment of the ErbB transgenic strains, SAA4M-1M and SAA7F-1M, with PD 163393 appeared to have no effect on the dorsalized chorion phenotype. By contrast, this compound increased the appearance of wild type chorions from 7.4% (untreated flies) to 22.33% in the SAA11F-1M strain and from 2.8% (untreated flies) to 18.8% in treated flies in strain SAB10F-1M. Similar to results obtained with CL-387,783, PD 163393 did not affect the chorion phenotype in the wild type strain (Fig. 16). PD 163393 has specificity for the ErbB2 receptor, but both treated and untreated flies from the ErbB2 transgenic strain laid eggs with severely dorsalized phenotype (Data not shown).

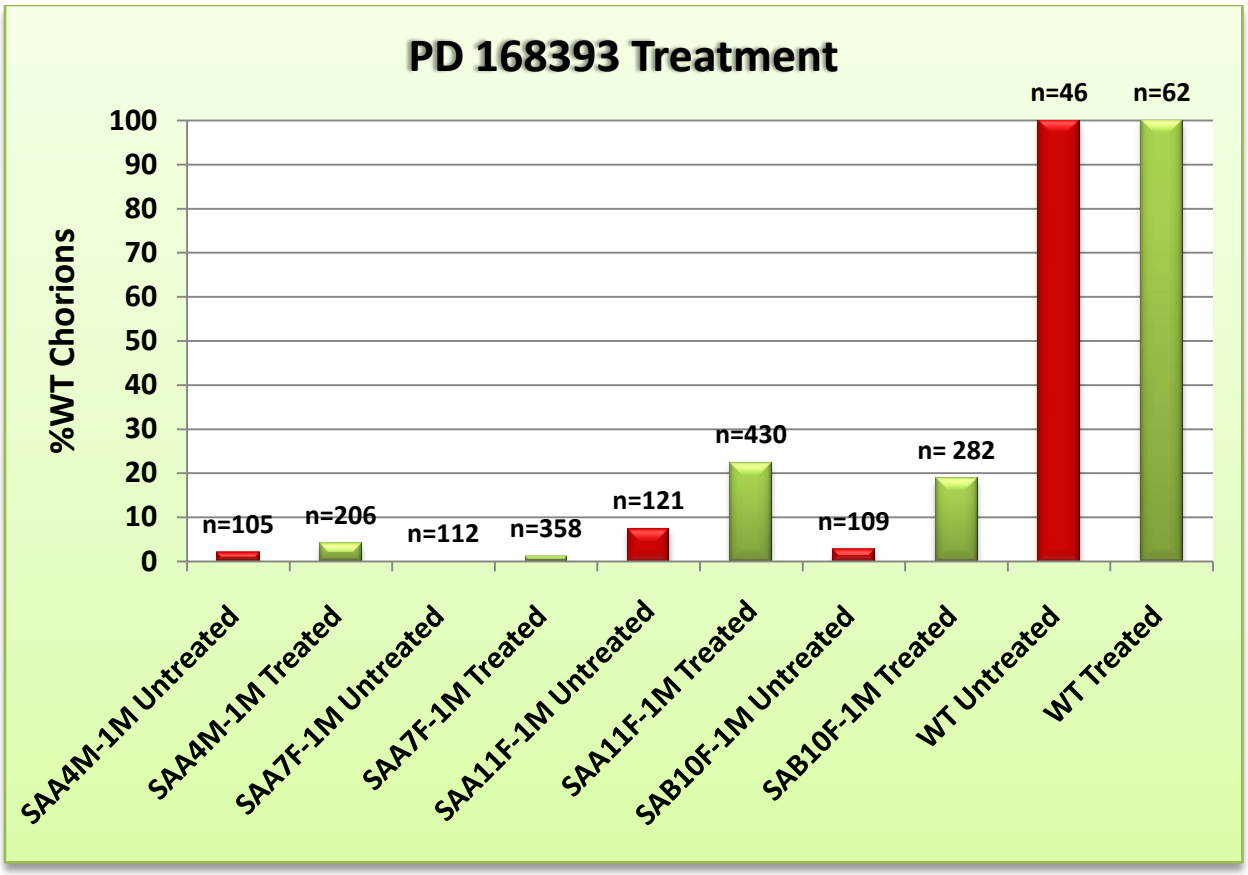


Figure 16: Effect of PD 163393 (1:100 dilution) treatment on ErbB1 and wild type *Drosophila* strains (n=total number of chorions)

Discussion

Aberrant signaling by the ErbB receptor family is a common feature of multiple types of human malignancies. Designing novel therapeutics to specifically target these receptors may provide better treatments and optimize patients' outcome. Since early validation of drug efficacy is a crucial step in the drug discovery process, a whole animal, *in vivo* screening approach may be a valuable tool to increase the rate of identification of higher quality leads. Transgenic *Drosophila* expressing properly localized and active ErbB receptors can potentially be utilized as a model system to screen for novel therapeutics for ErbB based cancers.

In the current study, thirteen transgenic *Drosophila* strains containing the human ErbB1 or ErbB2 genes were successfully generated and the chromosome position of each insert was mapped (Table 4&5). Subsequent misexpression experiments with the CY2 GAL4 driver indicated a proper localization of the two receptors. Since the ErbB receptors are transmembrane proteins they should be localized to the cell membrane. Because CY2 GAL4 drives protein expression during oogenesis, stage 10 egg chambers were examined for receptor expression and localization pattern. GFP expression in egg chambers from ErbB1 or ErbB2 transgenic strains was confined to the follicle cells and appeared to be localized to the cell membrane (Fig.12). This suggests that the GFP-tag does not interfere with the proper receptor expression and transport to the cell membrane.

The receptor expression levels were relatively the same across the ErbB1 and ErbB2 transgenics, but the strain containing the fly EGFR receptor (DER) had significantly lower GFP expression, although it was still membrane localized. In addition, only distinct patches of follicle cells appeared to strongly express GFP. These data suggest that DER is probably endocytosed and targeted for degradation through recruitment of the ubiquiting ligase Cbl. *Drosophila* has a single Cbl protein, D-Cbl, which is homologous to the mammalian c-Cbl. The human Cbl proteins can interact with the ErbB receptors by directly associating their PTB-binding domains with distinct C-tail phosphotyrosine residues. In addition, Cbl can bind to and utilize Grb2 as an adaptor protein to associate with the active receptors (Sorkin & Goh, 2009). Although *Drosophila* has a homolog of the human adaptor Grb2, called Drk, D-Cbl cannot bind to that adaptor protein. This is due to the fact that D-Cbl cannot interact with SH3-domain containing protein since it is C-terminally truncated. However, D-Cbl is still able to associate with activated DER, possibly through direct binding to distinct receptor phosphotyrosine residues. If these binding sites are not conserved from flies to humans, this may suggest that the human receptors in transgenic *Drosophila* cannot be ubiquitinated by D-Cbl and, therefore, may be able to escape the lysosomal degradation pathway following endocytosis (Hime et al., 1997).

Based on previous work in the Duffy Lab by C. Leduc, conserved phosphotyrosine residue binding sites were identified across the human ErbB receptors and the fly receptor. It was determined that both ErbB1 and ErbB2 contain two binding sites for the adaptor protein

Grb2 and one for the Shc adaptor (Fig. 17). Thus, it was hypothesized that the human ErbB1 and ErbB2 receptors would be able to activate downstream signaling pathways in *Drosophila.* Consistent with this hypothesis, functional analysis of ErbB1 and ErbB2 in transgenic *Drosophila* strains indicate that both receptors have activity.

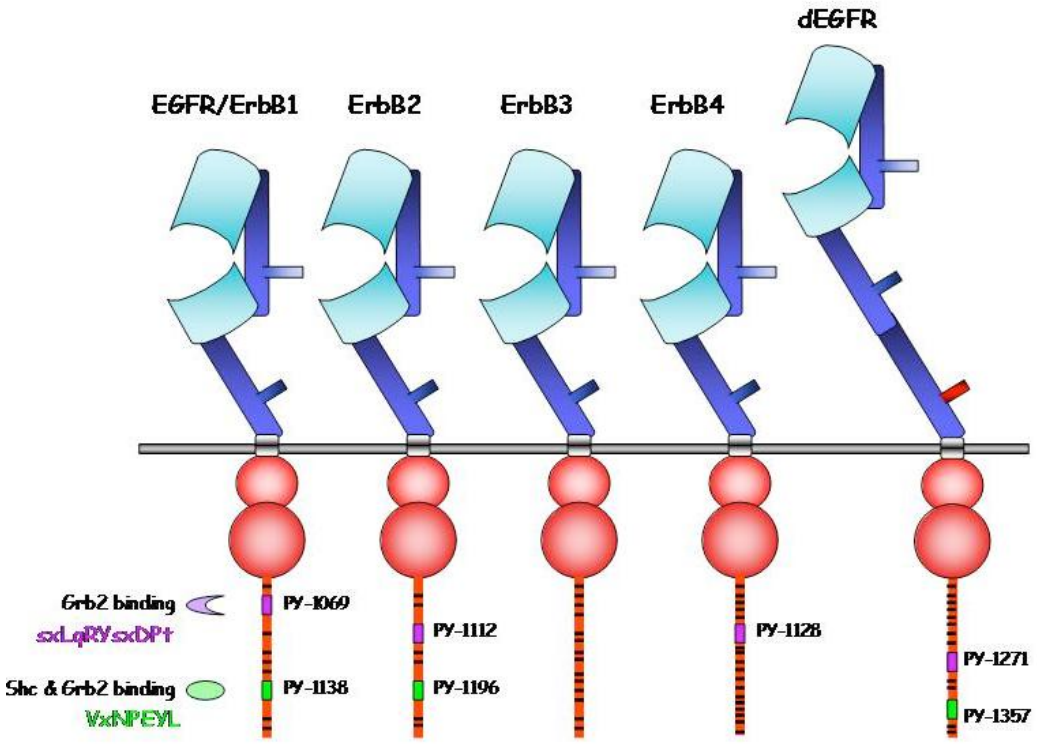


Figure 17: Conserved tyrosine residues at the C-terminal tails of the ErbB and DER receptor (Taken from: C. Leduc, 2007)

Misexpression of ErbB1 and ErbB2 during oogenesis resulted in a dorsalized chorion phenotype as shown in Fig. 13. The ability of the human receptors to produce the same phenotype caused by overexpression of the fly receptor indicates that ErbB1 and ErbB2 must be capable of associating with *Drosophila* adaptor proteins and activating the MAPK signaling cascade.

Since ErbB1 and ErbB2 are properly localized to the cell membrane and have activity in flies, it seems feasible to consider using transgenic *Drosophila* in an *in vivo* assay to identify novel therapeutic agents for ErbB-based cancers. Towards this goal, a drug screening assay was set up, in which two known small molecule tyrosine kinase inhibitors, CL-387,783 and

PD 163393, were tested for an ability to suppress signaling by ErbB1 and ErbB2 in transgenic flies overexpressing the receptors during oogenesis. Since a dorsalized chorion phenotype is indicative of receptor activity, reversion of this phenotype to wild type would be a sign of ErbB signaling inhibition. CL-387,783 treatment increased the appearance of wild type chorions in all four of the ErbB1 strains that were tested. After treatment, higher percent wild type chorions was observed in strains SAA11F and SAB10F as compared to the other two strains. Although SAA11F and SAB10F exhibit a strong dorsalized phenotype, it is less consistent as demonstrated by the presence of small amounts of wild type eggs even in the untreated strains.

Treatment with inhibitor PD 163393 increased the appearance of wild type chorionsin in both SAA11F and SAB10F, but had no effect on the SAA4M-1M and SAA7F-1M strains. The ErbB1 strain SAA7F-1M exhibited a severe dorsalized phenotype, which was also very consistent. A more robust reversion of this severely dorsalized chorion phenotype to wild type may require higher inhibitor concentrations and longer treatment periods. The ErbB2 transgenic strain ARA15F had the most severe chorion phenotype, as demonstrated by a characteristic peanut shape. Again, the severity of the phenotype may explain why PD 163393 had no effect on this strain, although this inhibitor has been shown to have specificity to the ErbB2 receptor as well.

The two small molecule tyrosine kinase inhibitors were also tested on wild type *Drosophila* strains. If the compounds were able to inhibit the basal signaling levels of the endogenous DER receptor, then the treated wild type flies would be expected to lay ventralized eggs. However, both the treated and untreated wild type strains laid only wild type eggs. This suggests that CL-387,783 and PD 163393 were not inhibiting the endogenous DER receptor and that the increased appearance of wild type eggs in the ErbB1 strains was due to specific inhibition of this receptor. Together, these data indicate that it is feasible to utilize transgenic *Drosophila* to screen for therapeutics that specifically block ErbB signaling.

The ErbB1 and ErbB2 transgenic *Drosophila* strains can also be utilized in high-throughput screens of libraries of compounds. However, it is important to first optimize the current assay conditions. One particular problem in this screening assay is the drug delivery

approach. A small amount of inhibitor solution was pipetted on top on the solidified apple juice agar in each well. However, using this delivery method, whether the flies are actually eating the food containing the inhibitor and relative dosing cannot be easily assessed. Consequently, it becomes difficult to assess if absence of an effect is really due to poor drug efficacy or dosing.

One solution to the delivery approach would be to mix the apple juice agar with food coloring. A better alternative, however, would include a tool that would allow precise measurement of ingestion. One such tool, the Capillary Feeder (CAFE) assay, described by William W. Ja et al., uses graduated glass microcapillaries to deliver liquid food to flies kept in small chambers. This method allows for precise quantification of actual ingestion in individual *Drosphila* flies and is, therefore, an excellent tool to monitor oral drug delivery. The Capillary Feeder also circumvents the need to add supportive materials into the food such as coloring or agar (Ja et al., 2007). Combining the Capillary Feeder with the drug screening assay described in the current study can potentially produce a useful tool to identify or validate novel therapeutics targeting the ErbB receptor family. Since drug intake with the CAFE assay can be continuously monitored and quantified, a presence or absence of a drug effect can be more strongly correlated to the efficacy of the given compound.

The positive results obtained with the *in vivo* tyrosine kinase inhibitors screen demonstrated the potential of transgenic *Drosophila* as a model organism for drug discovery. The incorporation of transgenic *Drosophila* drug screens with sophisticated drug delivery tools into the early stages of the drug discovery process may provide a potential means to enhance the efficiency of early drug validation, and thus help to streamline the drug discovery process. In addition, *Drosophila* represents a simple *in vivo* model system to study the complex interaction of the human ErbB receptors. Strains expressing combinations of human ErbB receptor could be useful models to study specific receptor interactions. For instance, the ErbB2-ErbB3 heterodimer is the most mitogenic receptor complex and is implicated in a number of cancers. Studying the ErbB family in a simple model system can help gain insight into signaling mechanisms and, subsequently, provide a rationale for the development of novel targeted cancer therapies.

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